ENVIRONMENTALLY RELEVANT CONCENTRATIONS OF AMMONIUM PERCHLORATE INHIBIT THYROID FUNCTION AND ALTER SEX RATIOS IN DEVELOPING *XENOPUS LAEVIS*

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Abstract—Embryos and larvae of the South African frog *Xenopus laevis* were exposed to ammonium perchlorate (AP) or control medium for 70 d. The dosage levels (59 ppb, 14,140 ppb) bracketed a range of perchlorate concentrations measured in surface waters at the Longhorn Army Ammunition Plant (LHAAP) in Karnack, Texas, USA. The experiment also included a 28-d non-treatment recovery period to assess the reversibility of AP effects. There were no significant effects of AP on mortality or hatching success. There were no effects of AP on developmental abnormalities such as bent/asymmetric tails or edema. Ammonium perchlorate inhibited forelimb emergence, the percentage of animals completing tail resorption, and hindlimb development during the 70-d exposure period. Only the upper AP concentration reduced whole-body thyroxine content, whereas both concentrations caused significant hypertrophy of the thyroid follicular epithelium. Both concentrations of AP caused a skewed sex ratio, significantly reducing the percentage of males at metamorphosis. The effects of AP on metamorphosis and thyroid function were reversed during the 28-d nontreatment recovery period. We conclude that AP inhibits thyroid activity and alters gonadal differentiation in developing *X. laevis*. These effects were observed at concentrations at or below concentrations reported in surface waters contaminated with ammonium perchlorate, suggesting that this contaminant may pose a threat to normal development and growth in natural amphibian populations.

Keywords—Amphibian   Thyroid   Perchlorate   Amphibian decline   Metamorphosis

INTRODUCTION

The importance of thyroid hormones in amphibian metamorphosis was first realized at the beginning of the last century, when Gudernatsch [1] demonstrated that feeding horse thyroid tissue to tadpoles accelerated metamorphosis. Since these early experiments, the critical role of thyroid hormones (TH) in initiating and coordinating the vast organismal changes that accompany metamorphosis have been firmly established [2]. In both anurans and urodeles, metamorphosis is accompanied by an increase in the synthesis and secretion of TH [3–5], an increase in iodide uptake by the thyroid gland [3,4], and in some species, an increase in thyroid follicle cell height [4], presumably reflecting epithelium stimulation by thyroid-stimulating hormone (TSH). The role of the thyroid gland in metamorphosis can be demonstrated by treating larvae with one of any number of pharmacological agents that block iodide uptake (e.g., perchlorate [6–9]), iodide coupling or coupling of iodothyronines (e.g , thiourea [9]), or deiodination (such as propylthiouracil [PTU] [10]). Chronic TH deficiency can lead to oversize larvae that continue to grow but fail to develop limbs and other adult structures [11].

Disruption of normal thyroid function during larval development can have permanent effects on the reproductive system in frogs. Thyroid hormone deficiency during larval development prevents masculinization of key secondary sex structures in male frogs, such as the development of laryngeal muscles involved in calling in the South African clawed frog *Xenopus laevis* [10]. Thyroid hormones may also be important for go-nadal differentiation in anurans [12]. Although a role for TH in gonadal differentiation is still debated, pharmacological blockade of TH synthesis can dramatically affect gonadal sex. Treatment of *X. laevis* with thiourea during larval development results in 100% females [13]. Similar treatment results in 100% males in another species, the common reed frog, *Hyperolius viridiflavus* [13]. In contrast, several studies suggest no role for TH in gonadal differentiation [13]. The variability in TH effects on gonadal differentiation undoubtedly has to do with the type and concentration of thyrostatic agent used and at what stage developmental exposure occurs.

Reports of declining amphibian populations have focused attention on the potential role of xenobiotic chemicals in disruption of thyroid function and metamorphosis in larval amphibians. One chemical of specific concern is ammonium perchlorate (AP), a compound widely used in the aerospace industry and by the military as an oxidizer in rocket fuels. Ammonium perchlorate is highly water soluble and degrades slowly in surface waters and sediments. Perchlorate levels ranging from 8 µg/L to 3.7 g/L have been reported in surface and ground waters at various places in the western United States [14]. Perchlorate levels as high as $31.2 \pm$ standard error 0.21 mg/L were reported in surface waters and sediments at Longhorn Army Ammunition Plant (LHAAP) [15]. Although levels of AP reported at LHAAP are an order of magnitude lower than those traditionally used to experimentally block metamorphosis, they are within a wide range of sublethal concentrations that reduce or prevent TH-dependent aspects of metamorphosis such as forelimb emergence (FLE), tail resorption, and hindlimb growth [16]. Whether environmentally relevant concentrations of AP result in measurable effects on

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thyroid function has not been studied. Furthermore, whether the effects of AP are reversible is not known. The goal of the present study was to examine the effects of environmentally relevant concentrations of AP on thyroid function in developing *X. laevis*. Whole-body TH content, height of the follicular epithelium, and thyroid volume were used to gauge AP effects on thyroid function. Because of previous work suggesting that THs are required for gonadal sex differentiation, we also examined AP effects on gonad differentiation. Portions of this work have previously appeared in abstract form [16].

METHODS

Reagents

Ammonium perchlorate (CAS 7790-98-9) with a purity of 99.999% was purchased from Aldrich Chemical (Milwaukee, WI, USA). L- [^{125}I]-thyroxine (T$_4$, specific activity = 969 Ci/mmol) was purchased from NEN Life Sciences Products (Boston, MA, USA).

Animals

Sexually mature male and female *X. laevis* imported from South Africa were purchased from *Xenopus* Express (Homosassa, FL, USA). Adults were maintained in dechlorinated water on a 12:12h light:dark regime (lights on 0700) and fed frog brittle (Nasco, Ft. Atkinson, WI, USA) three times weekly immediately following a water change. Water was changed three times each week.

Prior to breeding, adults were allowed to acclimate in 45-L glass aquaria containing 18 L frog embryo teratogenesis assay-*Xenopus* (FETAX) medium [17]: NaCl, 10.7 mM; NaHCO$_3$, 1.14 mM; KCl, 0.4 mM; CaCl$_2$, 0.14 mM; CaSO$_4$, 0.35 mM; MgSO$_4$, 0.62 mM. The FETAX medium was prepared using deioinzed water passed through a 1.2-ft^3 carbon filter immediately prior to use.

Naturally fertilized eggs were obtained from one or three to five pairs of adults. Spawning was artificially induced by injections of human chorionic gonadotropin (Sigma, St. Louis, MO, USA). Immediately following the last set of injections, males and females were placed together in 21-L glass breeding tanks with false bottoms made of silicone-coated 0.5-inch hardware mesh and left to breed overnight. Viable embryos were identified by visual observation with a binocular dissecting microscope. Embryos and tadpoles up to 5 d old were held in 9 L of FETAX medium or test solution in 21-L glass tanks acclimated to 22°C (±2°C) on a 12L:12D regimen. Five-day old larvae were transferred to 45-L glass tanks containing 18 L FETAX medium or test substance. Tadpoles were fed 0.4 g of powdered frog brittle (Nasco) mixed in 2 ml FETAX solution per tank every 72 h immediately following a 50% water change. All procedures involving *X. laevis* were approved by the Texas Tech Animal Care and Use Committee (Lubbock, TX, USA).

Analytical procedures

Ammonia, pH, and specific conductance of the tank water were monitored every 7 d. Dissolved oxygen was monitored every 2 d, and water temperature was monitored daily. A YSI® model 85 meter (Yellow Springs, OH, USA) was used to monitor water temperature, percent dissolved oxygen, specific conductivity, and salinity for each tank. Free ammonium ion and pH levels of the water in each tank were determined with a Hach® spectrophotometer model DR/2000 (Loveland, CO,

USA) and an Oakton® pH meter (Gresham, OR, USA), respectively.

The Cooperative Extension Service of the University of Georgia (Athens, GA, USA) performed a chemical analysis of the food and water. Adult and larval frog brittle contained no detectable pesticide residues (limits of detection ranged from 0.01 to 0.2 ppm for 22 different pesticides). Powdered frog brittle (larvae food) contained low levels of barium (12 ppm), arsenic (0.39 ppm), and selenium (0.45 ppm); adult frog brittle contained barium (7.6 ppm), arsenic (0.44 ppm), and selenium (1.03 ppm). Water analysis revealed no detectable pesticides or heavy metals.

Verification of AP in diluted source solutions was performed by ion chromatography [15,18]. Perchlorate analysis was carried out using a Dionex® DX-500 ion chromatography system equipped with a GP50 gradient pump, a CD20 conductivity detector, and an AS40 automated sampler (Dionex, Sunnyvale, CA, USA). PeakNet® chromatography software (Dionex) was used to control the system. A Dionex IonPac AS16 (250 × 4 mm) analytical column was used for ion separation. Conditions for the system were runtime = 12 min; flow rate = 1.0 ml/min; eluent = 100 mM sodium hydroxide; injection volume = 1,000 μl. Ion detection was by suppressed conductivity in the external water mode. Using the analytical method described above, the detection limit for perchlorate anion in water was 1 ppb. The lower limit of quantitation was 2.5 ppb.

Experimental design

Approximately 250 embryos (Nieuwkoop-Faber [NF] stages 4–10, [19]) were exposed to one of two concentrations of AP (target concentrations of 38 and 14,040 ppb) diluted in FETAX medium or FETAX medium alone for 70 d beginning <24 h after oviposition. Animals were then allowed to develop in normal FETAX medium for an additional 28 d to determine the reversibility of potential AP effects. All treatments were performed in duplicate in a total of three independent experiments that were conducted between October 1999 and April 2000. A 50% change of test and control solutions was performed every 72 h. Constant aeration was maintained throughout the experiment. The AP concentrations used represent high and low concentrations reported in April 1999 for effluent from the burning ground No. 3 groundwater treatment plant located at the LHAAP [20].

Sample collections were performed 6, 10, and 14 weeks (40, 68, and 96 d) posthatch. At 40 and 68 d posthatch, 10 larvae from each tank were weighed, staged, measured for total length, euthanized by immersion in 3-aminobenzoic acid ethyl ester (MS-222, 1 g/L in distilled water). Five animals were frozen on dry ice for subsequent determination of whole-body T$_4$ content while an additional five animals were fixed in 10% neutral buffered formalin for subsequent histological analyses. After the 68-d posthatch sampling, the remaining animals from each test and reference solution were transferred to treatment-matched 45-L glass aquaria and allowed to develop for an additional 28 d in untreated FETAX medium. After 28 d (day 96 posthatch), all remaining animals were weighed, staged, measured for total length, and euthanized by immersion in MS-222. Five euthanized animals from each tank were frozen on dry ice for subsequent T$_4$ determination while an additional five animals from each tank were fixed in 10% neutral buffered formalin for subsequent histological analyses. The remaining animals from each tank were placed in 10%

neutral buffered formalin for subsequent determination of gonadal sex.

Beginning on the day of hatch, hatching success (No. unhatched eggs/total No. embryos), percent deformities (No. showing bent tails, asymmetric tails/No. hatched), percent edema (No. showing distention of body with fluid/No. hatched), percent abnormal swimming (No. showing abnormal swimming/No. hatched), percent mortality (No. dead larvae/No. hatched), percent FLE (both forelegs visible), and percent metamorphosed animals (% completing tail resorption) were noted daily for each test and reference solution. Dead animals were removed and preserved in 10% neutral-buffered formalin. Snout–vent length, NF stage, hindlimb length (HLL), and total length were measured approximately every 5 d from 10 tadpoles per tank beginning 16 d after hatching.

Thyroid hormone extraction and radioimmunoassay

Tetraiodothyronine (T_4) was extracted from larvae and newly metamorphosed froglets using methods derived from a number of published reports [5,21,22]. Briefly, frozen whole tadpoles and froglets were weighed and then homogenized in 3 volumes of ice-cold methanol high-performance liquid chromatography (HPLC)-grade containing 1 mM propylthiouracil (PTU) using a Tekmar homogenizer. Homogenates were then sonicated. A trace amount (1,000 cpm) of [^{125}I]T_4 in methanol (containing 1 mM PTU) was added to each homogenate and the homogenates incubated at room temperature for 30 min. Homogenates were then centrifuged at 1,000 g for 20 min at 4°C. Supernatants were recovered, mixed with 2 vol $CHCl_3$, and TH back-extracted into 1 to 3 ml of NH_4OH (2 N) followed by centrifugation at 2,000 g for 15 min at 4°C. The aqueous phase was recovered and the back-extraction repeated twice, each time followed by centrifugation at 2,000 g for 15 min at 4°C. The aqueous fractions were pooled and evaporated in a Jouan centrifugal evaporator. Prior to radioimmunoassay, samples were purified by ion exchange chromatography as described by Mallol et al. [21]. Dried samples were reconstituted with 1 ml 2 N NH_4OH, mixed with 2 ml $CHCl_3$, and centrifuged at 2,000 g for 15 min at 4°C. The supernatants were collected and applied to Polyprep chromatography columns (Bio-Rad, Hercules, CA, USA) containing 1.5 ml AG 1-x 2 resin (200–400 mesh, chloride form, Bio-Rad) resin equilibrated with acetate buffer (pH 7). The columns were washed with 2 ml acetate buffer followed by a series of washes (2 ml each) designed to elute lipids and proteins from the resin, including 100% ethanol, pH 4 acetate buffer, pH 3 acetate buffer, 1% acetic acid, and 35% acetic acid. Eluates from these washes were discarded. Thyroid hormones were eluted from the column by passing six fractions (0.5 ml) of 75% acetic acid through the column. In our hands, T_4 eluted in fractions 3 through 5. The ion exchange columns were calibrated using [^{125}I]T_4. Column fractions containing T_4 were pooled, evaporated, reconstituted in radioimmunoassay buffer, and recoveries determined using a Cobra 5005 γ-counter (Packard, Downers Grove, IL, USA).

The T_4 content of reconstituted extract samples was determined by radioimmunoassay following MacKenzie et al. [23]. Aliquots (50 µl) of reconstituted extract were incubated in the presence of T_4 antiserum (1:200) and [^{125}I]T_4 for 90 min at 37°C followed by a 16-h incubation at room temperature. Assays were terminated by precipitation with goat antirabbit gamma globulin in ice-cold 5% polyethylene glycol.

The T_4 antibody used (T4-15, Endocrine Sciences, Calabasas, CA, USA) recognizes T_4 and, to a lesser degree, D-T_4 (69.6%) but exhibits low cross-reactivity with T_3 (3.7%) and diiodothyronine (<0.02%). Intraassay variance was 3.7% (n = 8). T_4 content in samples was determined using a four-parameter logistic transformation of [^{125}I]T_4 displacement by authentic T_4 standards. The assay was validated by comparing displacement of [^{125}I]T_4 by serially diluted extracts with that produced by authentic T_4 standards.

Histological assessment of thyroid activity

Tadpole heads were dehydrated in a graded series of alcohols and processed for routine paraffin embedding. Serial transverse sections (7 µm) through the head were mounted on glass slides and stained using Harris's progressive hematoxylin and eosin procedure and coverslips were mounted. Follicle epithelial cell height and right thyroid gland volume were measured using an Olympus BH-2 compound microscope equipped with a Sony CCD/RGB video camera (Tokyo, Japan) and monitor and a CompuAdd 450DX2 computer with Image Pro (Media Cybernetics, Silver Spring, MD, USA) imaging software. The software was calibrated using a Bausch & Lomb calibration slide (Rochester, NY, USA) prior to recording. The calibration was saved to the computer hard drive to ensure consistent measurements over time. Epithelial cell height measurements were taken from five randomly chosen cells in five sections of the right thyroid gland for each specimen ($n = 25$ observations/animal). A single mean value for epithelial cell height was then calculated for each animal. The cross-sectional area of all serial sections through the right thyroid gland for each animal was measured and summed. Total cross-sectional area (mm^2) × section thickness (7 µm) [6] was used to calculate the right thyroid gland volume for each animal.

Gonadal sex determination

Sex ratios were determined by direct visual inspection of approximately 266 to 286 animals per treatment. Previously fixed specimens were rinsed in deionized water and pinned to a dissecting dish of hardened paraffin wax and placed under an Olympus SZ-PT binocular dissecting microscope with a Fiber-Lite series 180 high-intensity illuminator (Tokyo, Japan) as a light source. The abdominal cavity was opened and the intestines removed. The kidneys were located in the retroperitoneal region. Gonads appeared as thin whitish strips of tissue on the medial side of each kidney. Ovaries were long and lobular, with small areas of dark pigmentation visible. Testes were shorter and lacked both lobes and pigmentation. Results were validated by histological examination of gonads from four visually sexed animals (one male and one female at NF stages 54 and 56) from each concentration.

Data analysis

Differences in HLL were tested by one-way analysis of variance (ANOVA) followed by the Tukey–Kramer multiple comparisons test. The statistical analyses of %FLE and percent complete tail resorption were complicated by zero values for some of the 70-d time points. In these cases, we used a nonparametric (Kruskal–Wallis ANOVA) test followed by Dunn's multiple comparisons test. For all other data, we used parametric ANOVA. Sex ratios were analyzed by Chi-square. All statistical analyses were performed using InStat software (GraphPad, San Diego, CA, USA).

Table 1. Statistical analysis of mean ($\pm$ SE) forelimb emergence (%), percent completing tail resorption (%), and snout–vent length (mm) in *Xenopus laevis* tadpoles exposed to ammonium perchlorate for 70 d[a]

Perchlorate (ppm)	Forelimb emergence		Tail resorption		Snout–vent length		
	70 d[b]	98 d[c]	70 d	98 d	42 d[d]	70 d	98 d
0.002 ± 0.001[e]	26.0 ± 11.6	66.7 ± 8.7	16.6 ± 10.2	42.1 ± 9.59	8.30 ± 0.69	10.4 ± 1.04	18.9 ± 1.47
0.059 ± 0.005	2.72 ± 1.31	45.6 ± 3.7	0.82 ± 0.50	$14.7 \pm 2\,11$*	7.72 ± 0.94	$10\,6 \pm 0.37$	14.1 ± 2.38
14.14 ± 0.348	0.00 ± 0.00*	41.6 ± 5.5*	0.00 ± 0.00*	1.64 ± 0.68*	7.59 ± 0.86	10.7 ± 0.84	15.8 ± 0.56

[a] Data are mean $\pm$ SE of measurements from one to two replicates per treatment from three independent experiments. Asterisks indicate significant difference from control ($p < 0.05$) based upon Kruskal–Wallis analysis of variance (ANOVA) followed by Dunn's multiple comparisons test (percent forelimb emergence, percent completing tail resorption) or one-way ANOVA followed by the Tukey-Kramer multiple comparisons test (snout–vent length).
[b] Sampled 70 d after beginning of exposure.
[c] Sampled after a 28-d nontreatment recovery period.
[d] Sampled 42 d after beginning of exposure.
[e] Control.

RESULTS

Analysis of perchlorate in dosing solutions revealed that the actual concentrations (2 ppb, 59 ppb, 14.1 ppm) were close to the target concentrations of 0 ppb, 38 ppb, and 14 ppm (Table 1). There was a trace amount of perchlorate in the control exposure, although six replicates across three experiments averaged less than the lower limit of detection for the assay (2.5 ppb). Embryo quality was acceptable in all treatments as determined by mean hatching success (79% for controls, 81% for 59-ppb group, 78% for 14.1-ppm group), 5-d mortality (5.8% for controls, 6.9% for 59-ppb group, 9.1% for 14.1-ppm group), and incidence of edema at 5-d posthatch (0.5% for controls, 0.9% for 59-ppb group, 1% for 14.1-ppm group). The affects of AP on %FLE, % completing tail resorption, and SVL are shown in Table 1. There was a significant concentration-dependent effect of AP on %FLE at 70 ($KW = 11.5$, $p = 0.0002$) and 98 d ($F = 4.4$, $p = 0.0327$). There was no forelimb emergence in the 14.1-ppm treatment group at the end of the exposure period (70 d, Table 1). Tadpoles exposed to 14.1 ppm AP were released from thyrostasis when transferred to FETAX solution for the 28-d nontreatment recovery period, as %FLE increased from 0 to 46% between day 70 and day 98. The animals did not fully recover within this 28-d window, as %FLE was still statistically lower than controls at 98 d (Table 1). A similar trend was observed in the 59-ppb treatment group, as there was a 17-fold increase in %FLE during the recovery period (compare 70 d vs 98 d). In contrast, controls exhibited just a 2.6-fold increase in %FLE during the recovery period. There were significant concentration-related effects of AP on tail resorption at both the 70- ($KW = 11.6$, $p = 0.0002$) and 98-d ($F = 12.7$, $p = 0.0007$) sampling points (Table 1). The percentage of animals completing tail resorption in the 59-ppb AP group rose 18-fold compared, with just a 2.5-fold increase in controls during the recovery period. As with %FLE, this rebound was not entirely completed within the recovery period, as the percentage of animals completing tail resorption was statistically lower for both AP concentrations compared with controls at 98 d.

The inhibitory effects of AP on HLL were robust. One-way ANOVA revealed a significant treatment effect at both 70 d ($F = 17.3$, $p = 0.0002$) and 98 d ($F = 5.5$, $p = 0.0174$). The effects of AP on HLL over time are shown graphically in Figure 1 for each 5-d sampling date starting at day 16 posthatch and ending on experimental day 98. The statistical analysis of AP effects on HLL over time is summarized in Table 2. Both AP concentrations significantly inhibited hindlimb growth

throughout the 70-d treatment period. As with other TH-dependent indices of metamorphosis, there was a concentration-dependent increase in HLL during the recovery period (Table 2). Hindlimb growth increased 24- and 7-fold in the 14.1-ppm and 59-ppb treatment groups, respectively, during the recovery period, compared with just a 2.5-fold increase in hindlimb growth for controls. These effects are especially dramatic given that hindlimb growth increased just 2.9- and 18-fold in the 14.1-ppm and 59-ppb treatment groups during the exposure period. In contrast, controls exhibited a 27-fold increase in HLL during the same period. The effects of AP on growth were specific for HLL, as no treatment-related effect on SVL was observed (Table 1).

The effects of AP on T_4 concentrations and total content are summarized in Table 3. Serial dilutions of whole-body

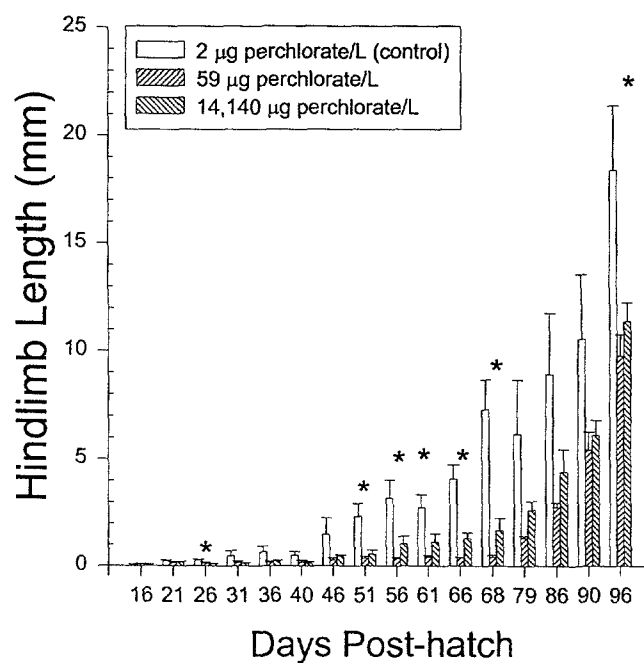

Fig. 1. Effects of ammonium perchlorate (AP) on hindlimb growth over time in larval *Xenopus laevis* Asterisks indicate significant variation among treatment groups as assessed by one-way analysis of variance (ANOVA). Bars represent the mean + standard error of mean (SEM) of five to six replicates per group. Each replicate represents an average of 10 tadpoles except for the 96-d posthatch sampling, in which case each replicate represents all animals remaining in the tank at the end of the experiment.

Table 2. Statistical analysis of mean (± SE) hindlimb length (mm) in *Xenopus laevis* larvae exposed to ammonium perchlorate (AP) for 70 d[a]

Sampling day[b]	Perchlorate (ppm)			F score	P-Value
	0 002 ± 0.001[c]	0.059 ± 0.005	14 14 ± 0.348		
26	0.27 ± 0 04	0 09 ± 0 03*	0 14 ± 0 05	4 26	0.0359
51	2.34 ± 0 59	0.55 ± 0 20*	0 37 ± 0 08*	8.48	0.0039
56	3.18 ± 0 86	1.03 ± 0 37*	0 35 ± 0 07*	7 32	0.0067
61	2.73 ± 0 61	1.12 ± 0 38*	0 43 ± 0 07*	8 10	0.0046
66	4.07 ± 0.66	1.29 ± 0 26*	0.39 ± 0 02*	21 1	<0 0001
68	7 29 ± 1.38	1.66 ± 0.58*	0 41 ± 0 10*	17 4	0.0002
96	18.4 ± 3.04	11.4 ± 0 86	9.77 ± 1 00*	5.37	0.0186
% Increase					
Posthatch day 26–68	27	18	3		
Posthatch day 68–96	3	7	24		

[a] Asterisks indicate significantly different from control based on one-way analysis of variance followed by the Tukey–Kramer multiple comparisons test. Larvae were exposed to AP for 68 d posthatch Larvae were then transferred to plain frog embryo teratogenesis assay-*Xenopus* medium until day 96 posthatch.
[b] Posthatch sampling day.
[c] Control.

extracts paralleled authentic T_4 standards in the radioimmunoassay (Fig. 2). Whole-body T_4 concentration and total content was reduced in the 14.1-ppm treatment group at 70 d (Table 3). Only whole-body T_4 content was affected by AP at 98 d. The effects of AP on thyroid histology are shown in Table 4. Both concentrations of AP caused a statistically significant increase in thyroid epithelial cell height at 70 d but not at 42 or 98 d. Thyroid gland volume was not affected by AP (Table 4). Analysis of gonadal sex ratios is shown in Table 5. The sex ratio was skewed in both AP treatment groups compared with controls. Exposure to AP significantly reduced, in a concentration-dependent manner, the percentage of males at metamorphosis (Table 5).

DISCUSSION

Our data indicate that concentrations of AP at or below concentrations measured at LHAAP inhibit a number of TH-dependent indices of development and metamorphosis in *X. laevis*. There were no significant AP-related effects on mortality or hatching success at the concentrations used in the present study, consistent with our previous findings [16]. The percentages of animals reaching FLE and completing tail resorption increased during the recovery period, suggesting that the effects of AP exposure were reversible. Likewise, there was a concentration-dependent increase in hindlimb growth during the recovery period (Table 2). The finding that AP inhibits TH-dependent aspects of metamorphosis and growth in a reversible fashion is consistent with the only known mech-

anism of perchlorate action, inhibition of thyroidal iodide uptake [24]. Perchlorate ions competitively inhibit iodide binding to the sodium-dependent iodide symporter (NIS) and, as a result, reduce the amount of iodide available for TH synthesis. Whether perchlorate is actually transported by the NIS is still a matter of debate. A recent study on the cloned rat NIS expressed in *X. laevis* oocytes reported that perchlorate binds to the transporter but is not transported across the cell membrane [25]. In contrast, a number of studies have shown that $^{36}ClO_4^-$ is actively taken up by the thyroid gland [26–29]. The $^{36}ClO_4^-$ is actively transported across the basal membrane into the cell and then transported into the follicular lumen, possibly by a facilitated transport process [28]. Peak uptake of perchlorate by the thyroid gland occurs within 4 to 6 h [29]. In some mammalian species, perchlorate can accumulate in the thyroid at levels approaching those observed for iodide [28,29]. Nonetheless, perchlorate that accumulates in the follicular lumen would not be available to inhibit iodide transport. How quickly perchlorate is eliminated in developing *X. laevis* is not known, but the present findings suggest that TH synthesis has partially recovered during the recovery period.

Because of the small size of the *X. laevis* larvae and the difficulties associated with collecting sufficient amounts of plasma, we chose to examine whole-body T_4 content in larvae during the exposure period and at the end of the recovery period. Serial dilution of ion exchange purified extracts displaced [^{125}I]T_4 identically to that of synthetic T_4 standards (Fig. 2). Although we did not determine the contribution of different

Table 3. Statistical analysis of mean (± SE) whole-body thyroxine (T_4) in *Xenopus laevis* tadpoles exposed to ammonium perchlorate for 70 d[a]

Perchlorate (ppm)	42 d[b]		70 d[c]		98 d[d]	
	T_4 (ng)	T_4 (ng/g)	T_4 (ng)	T_4 (ng/g)	T_4 (ng)	T_4 (ng/g)
0.002 ± 0 001[c]	0.35 ± 0.10	1.35 ± 0 55	1 03 ± 0 18	1 01 ± 0 29	2 65 ± 0 47	1 80 ± 0 21
0 059 ± 0 005	0.44 ± 0.19	1.26 ± 0.30	0.86 ± 0 29	0.79 ± 0 21	1 03 ± 0 23*	1.58 ± 0.25
14.14 ± 0.348	0.36 ± 0.05	1.80 ± 0.62	0.23 ± 0 07*	0.39 ± 0 14*	0.81 ± 0 13*	1.19 ± 0 13

[a] Values for 42 and 70 d reflect the means of five tadpoles pooled per sample taken from three experiment replicates. Values for 98 d represent measurements from individual tadpoles. Asterisks indicate significantly different from control ($p < 0 05$) based on one-way analysis of variance followed by the Tukey–Kramer multiple comparisons test.
[b] Sampled 42 d after beginning of exposure.
[c] Sampled 70 d after beginning of exposure.
[d] Sampled after a 28-d nontreatment recovery period.
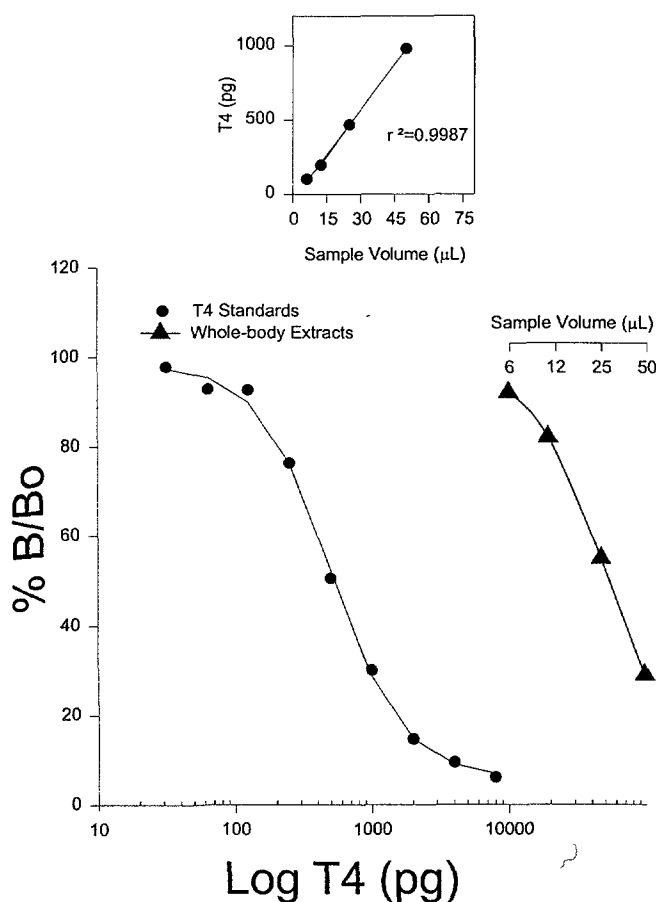[e] Control.

Fig. 2. Measurement of T_4 in serial dilutions of whole-body extracts showed parallelism with authentic T_4 standards.

Table 5. Gonadal sex in *Xenopus laevis* after exposure to ammonium perchlorate for 70 d[a]; ± SE—statistical analysis of mean

Perchlorate (ppm)	% Male	% Female	χ^2	p-Value
0.002 ± 0001[b]	42	58	3.41	$p = 0.06$
	($n = 120$)	($n = 166$)		
0 059 ± 0.005	33	67	16 7	$p < 0 0001$
	($n = 93$)	($n = 191$)		
14.14 ± 0 348	26	74	31.7	$p < 0.0001$
	($n = 69$)	($n = 197$)		

[a] Sample sizes are indicated in parentheses Chi-square statistic was calculated based on frequency deviation from a sex ratio of 50:50.
[b] Control.

had no effect on whole-body T_4, suggesting that whole-body TH measurements may not be the most sensitive indicator of AP exposure in developing anurans.

An indirect consequence of inhibiting TH synthesis is that secretion of TSH is released from negative feedback inhibition, leading to a pronounced enlargement of thyroid follicle cell size. This is precisely what was observed in both AP exposure groups at the end of the 70-d exposure. In both AP exposure groups, epithelial cell height was double that of the control group at 70 d. This effect was reversed during the recovery period (Table 4). A similar phenomenon was observed in rats fed AP via the drinking water for 90 d, although these authors utilized a 30-d rather than a 28-d nontreatment recovery period [31]. Interestingly, there were no statistically significant changes in volume of the thyroid gland, as previously reported for *Bufo arenarum* after perchlorate exposure for three or five months [6]. One reason for the discrepancy may be differences in the concentration of perchlorate to which the developing animals were exposed. Miranda et al. [6] exposed developing tadpoles to 0.034% $KClO_4$ (340 mg/L), a concentration 24-fold greater than the highest AP concentration used in the present study. Furthermore, although Miranda et al. [6] controlled for stage-dependent differences in thyroid volume, they did not control for differences in animal size or age. Larvae exposed to perchlorate for five months were compared with unexposed animals at the same developmental stage but not the same age [6]. Our data (Table 4) clearly show that thyroid gland volume changes not only as a function of age but also as a function of the animal's size. Larger animals had a greater thyroid gland volume per mg body weight than did smaller

tissue compartments to changes in whole-body T_4, Niinuma et al. [30] demonstrated that removal of the thyroid gland does not appreciably affect whole-body T_3 or T_4 in toad tadpoles, suggesting that whole-body T_4 mainly reflects extra-thyroidal T_4. The fact that 14.1-ppm-treated larvae had lower whole-body T_4 content is consistent with perchlorate inhibition of iodide uptake. Subchronic (90 d) exposure to AP via the drinking water reduced circulating T_4 and T_3 but elevated plasma TSH in rats [31]. Despite dramatic effects on TH-sensitive indices of metamorphosis, the lower exposure concentration

Table 4. Thyroid histology in *Xenopus laevis* tadpoles exposed to ammonium perchlorate for 70 d[a]

| Perchlorate (ppm) | 42 d[b] | | 70 d[c] | | 98 d[d] | |
	Cell height[e] (μm)	Volume[f] (mm^3/g)	Cell height (μm)	Volume (mm^3/g)	Cell height (μm)	Volume (mm^3/g)
0.002 ± 0.001[g]	4.15 ± 0.32	3.94 ± 1.14	5.88 ± 0.56	9.45 ± 2.99	9.08 ± 0.80	34 0 ± 8 34
	($n = 16$)	($n = 16$)	($n = 13$)	($n = 12$)	($n = 13$)	($n = 12$)
0.059 ± 0.005	4.51 ± 0 37	2.50 ± 0.57	9.32 ± 0.86*	22 4 ± 7.10	9 00 ± 0.82	21 0 ± 5 88
	($n = 15$)	($n = 14$)	($n = 18$)	($n = 18$)	($n = 13$)	($n = 13$)
14.14 ± 0.348	4.36 ± 0.51	2.80 ± 0.63	9.51 ± 1.01*	11 3 ± 2 80	10 1 ± 1 25	34 2 ± 13 6
	($n = 16$)	($n = 18$)	($n = 14$)	($n = 14$)	($n = 12$)	($n = 12$)

[a] Values are the statistical analysis of mean (± SE). Sample sizes are indicated in parentheses Asterisks indicate significantly different from control ($p < 0.05$) based on one-way analysis of variance followed by the Tukey–Kramer multiple comparisons test
[b] Sampled 42 d after beginning of exposure.
[c] Sampled 70 d after beginning of exposure.
[d] Sampled after a 28-d nontreatment recovery period.
[e] Cell height is mean (± SEM) follicle cell height measured from 25 to 30 cells per animal from the right thyroid gland
[f] Thyroid gland volume is mean ± SEM of cross-sectional area (mm^2) × length (mm) of consecutive serial sections through the right thyroid gland per gram body weight
[g] Control

larvae (Table 4). Collectively, our findings suggest that epithelial cell height is the most sensitive indicator of AP exposure, as maximal differences from the control were seen in the 59-ppb-treated animals.

In mammals, the developing testes express multiple thyroid hormone receptor (TR) isoforms, including TRα1 and TRα2 in fetal and adult human testes [32] and TRα1, TRα2, and TRβ in developing rat testes [33]. The precise role of TR in testes development is unknown, although neonatal hypothyroidism is associated with decreased Sertoli cell number, spermatogenic arrest at puberty, and reduced fertility [34,35]. In the present study, AP exposure resulted in a skewed sex ratio in favor of females, suggesting that disruption of thyroid activity impairs testes differentiation. These findings are consistent with but are not as robust as those previously reported by Hayes [13], who found that treatment of *X. laevis* with thiourea throughout the entire larval period completely prevented testes formation, producing 100% females. In contrast, Robertson and Kelley [10] reported no effect on gonadal differentiation when metamorphosis was blocked in *X. laevis*. There are a few possible explanations for these apparent discrepancies. First, Robertson and Kelley [10] treated larvae with PTU beginning at NF stage 54, after thyroid differentiation has already taken place [19]. Plasma T_4 is detectable in *X. laevis* by NF stage 54 [36]. Thus, the possibility exists that the developing gonad is exposed to low levels of T_4 prior to differentiation, which occurs by NF stage 56 [19]. Second, Robertson and Kelley [10] did not determine sex ratio but examined gonadal light and electron microscopic structure and reported that the testes of PTU-treated males had lower germ cell density and more connective tissue-like cells in the medulla. Our data suggest that the effects of AP on sex ratio are concentration dependent, and it is entirely possible that exposure to higher concentrations than reported here may lead to a completely skewed sex ratio such as reported by Hayes [13].

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